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SUGARS AND SUGAR PRODUCTS

Spectrophotometric Method for Hydroxymethylfurfural in Honey

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A new method is described for hydroxymethylfurfural (HMF) in honey; accuracy and precision are improved over the most used optical and chemical methods. With a clarified honey solution containing 0.1% sodium bisulfite as reference and a similar solution without bisulfite as sample, a difference spectrum is obtained which represents only the HMF in the sample, without the interfering absorption of the honey. The average recovery was 97.5% for 24 additions to honey of 0.8–40 mg HMF/100 g. Forty honey samples ranging from 0 to 40 mg/100 g were analyzed by 3 methods with the following average results: Winkler optical method, 7.25; Winkler chemical method, 4.83; and new bisulfite method, 5.05 mg HMF/100 g honey. Values by the latter 2 methods did not differ at the $P = 0.05$ significance level.

The presence in honey of hydroxymethylfurfural (2-hydroxymethyl-5-furaldehyde, HMF) was originally considered evidence of its adulteration with acid-converted invert sirup. Color tests were used for qualitative indication of its presence (1) but were soon criticized as it became known that authentic but strongly heated honey gave a positive test. A color test, with appropriate *caveat,* is still an official method (2). More recently, HMF content has been widely used as an indicator of the heating history of honey. Many European honey standards, including the Codex Alimentarius, set maxima for HMF in honey.

Recognition that HMF could be produced in appreciable amounts in honey stored at ambient temperatures, common in many honey-producing areas, has further complicated the use of HMF as an indicator of adulteration. A semilogarithmic relation has been demonstrated between the time needed for a honey to accumulate a given level of HMF and temperatures between 20 and 80°C (3). Differentiation between heat- or storage-abused honey and that containing added invert sugar requires accurate analytical methods.

Several general approaches have been used for quantitation of HMF for research and regulatory use: quantitation of earlier color tests (4, 5), Winkler's specific photometric chemical method (6), and Winkler's optical method using the prominent UV absorption band of HMF (6–8). Winkler's toluidine-barbituric acid method (6) has been adopted by the Codex Alimentarius; a comparison with his optical procedure indicated the former to be more reliable (3). Dhar and Roy (9) have criticized the optical method and described a charcoal column cleanup before analyses. This method is cumbersome and lengthy and not well suited for routine use.

Recent experience at this laboratory with the Winkler chemical method in the analysis of over 600 samples of honey has emphasized several shortcomings. In addition, a recent AOAC collaborative study of the 2 Winkler methods (10) has confirmed earlier observations (11) that the Winkler UV procedure produces anomalous results with certain honeys whose ultraviolet absorption spectra do not resemble those studied by Winkler. The results by the 2 methods differed significantly in the AOAC study (10). Interlaboratory precision was considerably better for the UV method than for the chemical procedure. However, the chemical procedure is considered to give more accurate values because of the specific nature of the reaction of HMF with barbituric acid and toluidine (6).

When the discrepancy became apparent, a random selection was made from the sample collection noted above and other samples available in this laboratory. The Winkler chemical method had been applied to all samples. Forty of these, representing negligible, low, moderate, and high contents of HMF, were then analyzed by the Winkler ultraviolet method. The results further confirmed the lack of agreement between the 2 methods.

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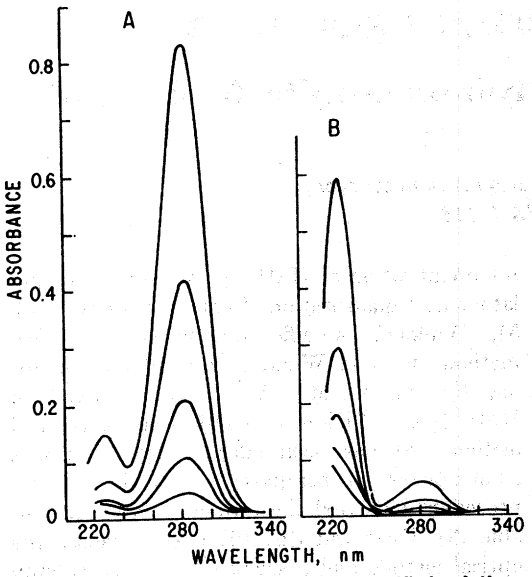
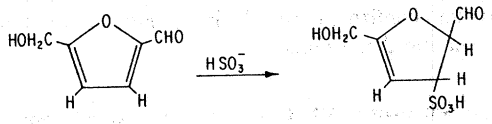


FIG. 1—Absorption spectra of Carrez-clarified solutions of hydroxymethylfurfural in A, water (vs. water); B, 0.1% $NaHSO_3$ (vs. 0.1% $NaHSO_3$). Concentrations: 6.26, 3.13, 1.57, 0.78, 0.31 μg HMF/mL.

The Winkler chemical method is now considered an undesirable procedure, however, because of the following major short-comings: It requires *p*-toluidine, a toxic reagent which carries a label warning of carcinogenicity to animals, and several AOAC collaborators objected to its use; and the color is unstable and temperature-dependent in its development.

A search was therefore instituted for a new procedure. An ideal method should retain the precision of the optical method and have the accuracy of the chemical method, without their deficiencies. The solvent extraction used in several other procedures should be avoided also.

This paper describes such a method, in which the UV absorbance of a clarified aqueous honey solution is determined against a reference solution of the same honey in which the 284 nm chromophore of HMF has been destroyed by bisulfite. The destruction of the 284 nm chromophore results from the classical addition of a nucleophile to the α,β-unsaturated carbonyl system.



Destruction of the chromophore eliminates the background absorption of the honey. The difference spectrum between sample (without bisulfite) and reference (with bisulfite) closely resembles the symmetric HMF absorption band between 250 and 330 nm (maximum 284) and is easily quantitated, using the literature value for the absorptivity of HMF.

METHOD

Reagents and Apparatus

(a) *Carrez solution I.*—Dissolve 15 g potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$) in water and dilute to 100 mL.

(b) *Carrez solution II.*—Dissolve 30 g zinc acetate ($Zn(CH_3CO_2)_2.2H_2O$) in water and dilute to 100 mL.

(c) *Sodium bisulfite* (*$NaHSO_3$*).—0.20% in water. Prepare fresh daily. Technical grade is adequate.

(d) *Spectrophotometer.*—Bausch and Lomb Spectronic 505 recording spectrophotometer was used. For routine analysis, spectrophotometer providing values at 284 and 336 nm is satisfactory.

Procedure

Transfer ca 5 g honey (weighed to 1 mg in small beaker) to 50 mL volumetric flask with total of 25 mL water. Add 0.50 mL Carrez solution I, mix, add 0.50 mL Carrez solution II, mix, and dilute to volume with water. A drop of alcohol may be added to suppress surface foam. Filter through paper, rejecting first 10 mL filtrate.

Pipet 5 mL filtrate in each of two 18 × 150 mm test tubes and pipet 5 mL water into one

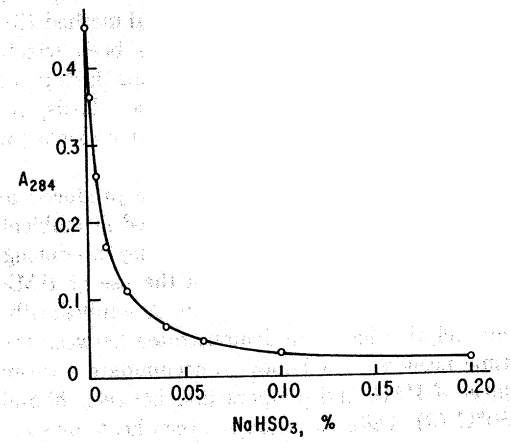


FIG. 2—Effect of bisulfite concentration on absorbance of hydroxymethylfurfural at 284 nm. Concentration, 3.42 μg/mL.

(sample) and 5 mL 0.20% bisulfite into the other (reference). Mix well (vortex mixer), and determine absorbance of sample against reference in 1 cm cells at 284 and 336 nm. If absorbance is too high for accuracy (>0.6), dilute sample solution as needed with water and the reference solution to the same extent with 0.1% $NaHSO_3$. Multiply absorbance values by appropriate dilution factor before calculation.

Calculation

$$\text{HMF (mg/100 g honey)} = (A_{284} - A_{336}) \times 14.97 \times 5/\text{sample}$$

$$\text{Factor} = \frac{126}{16830} \times \frac{1000}{10} \times \frac{100}{5} = 14.97$$

where 126 = mol. wt HMF;
16830 = molar absorptivity of HMF at 284 nm (12);
1000 = mg/g;
10 = centiliters/L;
100 = g honey reported;
5 = nominal sample weight.

Results and Discussion

The effect of bisulfite on the hydroxymethylfurfural (HMF) spectrum was studied. The UV spectra of equivalent concentrations of HMF in water and in 0.1% $NaHSO_3$ are shown in Fig. 1. The strong band at 284 nm is reduced by 94.3% in bisulfite and the 228 nm band is tripled in intensity.

The effect of bisulfite concentration on A_{284} for Carrez-clarified HMF solutions is shown in Fig. 2. Since bisulfite does absorb in the region of interest, the 0.1% concentration, which provides an acceptable compromise between effectiveness and interference, was chosen.

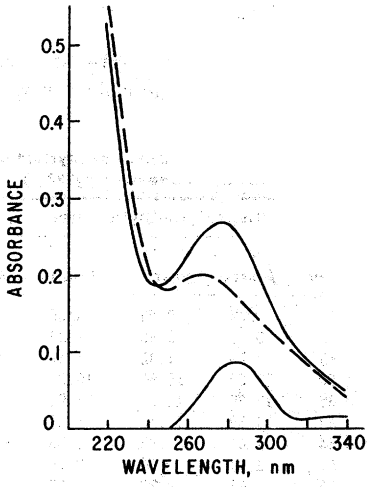


FIG. 3—Spectra of clarified 2% honey solutions in water (—) and in 0.1% bisulfite (---), and the difference spectrum with the former as sample and the latter as reference.

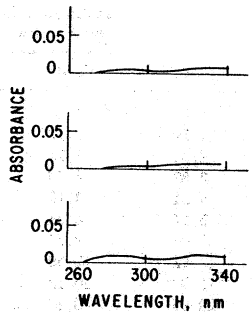


FIG. 4—Difference spectra as in Fig. 3 of 3 honeys with less than 0.05 mg HMF/100 g, measured by chemical procedure.

Figure 3 shows the absorption spectra of Carrez-clarified 2% honey solutions in water and in 0.1% bisulfite, each with the solvent as reference; the difference is also shown, recorded with the bisulfite solution as reference and the aqueous solution as sample. In addition to the HMF band, the difference spectrum shows nonspecific absorption in the 320–340 nm range. Figure 4 shows the difference spectra (as in Fig. 3) of honeys with no HMF by the Winkler chemical procedure (less than 0.05 mg HMF/100 g honey). That this general absorbance is not due to interaction of bisulfite with the monosaccharides was shown by examination of the spectrum of a solution of glucose and fructose at corresponding concentrations. As the bisulfite solutions stand for a few hours, the difference spectrum increases uniformly over the range 270–340 nm, but $A_{284} - A_{336}$ remains constant within 0.001–0.004. The difference spectrum passes below the baseline at 245 nm where absorbance by bisulfite becomes appreciable.

Since the use of difference spectra eliminates the contribution of honey constituents, sample size may be increased to obtain greater sensitivity, while the absorbance values remain within the optimal accuracy range of the instrument used. The Winkler optical method uses a 2% honey solution; a 5% solution is easily measured in this procedure.

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Table 1. Recovery of hydroxymethylfurfural added to honey[a] (mg/100 g honey)

Added	Total	Found	Rec., %
0.25	1.54	1.54	100.0
0.50	1.79	1.77	98.9
1.01	2.30	2.17	94.4
1.51	2.80	2.70	96.4
3.02	4.31	4.12	95.6
3.85	5.14	4.96	96.5
7.75	9.04	8.74	96.7
11.63	12.92	12.49	96.8
15.63	16.92	16.12	95.3
19.67	20.96	20.17	96.2
39.07	40.36	39.70	98.4

[a] Honey contained 1.29 mg HMF/100 g.

Recovery of Added HMF

Two series of recovery tests were performed: Small increments of HMF were added to aliquots of a honey solution; and single low and high amounts of HMF were added to each of a number of honeys selected for a variety of shapes of their UV absorption spectra.

Amounts of HMF indicated in Table 1 were added to aliquots of 25 ml of a 20% aqueous honey solution. These were analyzed as described above. Five replicate analyses of the stock honey (1.26, 1.30, 1.30, 1.27, 1.30 mg HMF/100 g) were averaged to obtain the base value for calculating recoveries. Per cent recovery is the amount found divided by the amount originally present plus the amount added. Recoveries were in the 95–100% range, averaging 96.9%, as shown in Table 1.

Six honey samples which showed considerable discrepancies in HMF content when determined by the Winkler chemical and Winkler UV methods were selected for the second recovery test. These are the first 6 samples in Table 3. Figure 5A shows the variety of shapes of the spectra in the 250–320 nm region, which accounts for the discrepancies between the 2 Winkler methods. Figure 5B shows the difference spectra for these samples. Each has a maximum at 284 nm. HMF amounts equivalent to about 1 and 10 mg/100 g honey were added to two 25 mL aliquots of a 20% solution of each. One aliquot was analyzed without added HMF. Each was clarified and analyzed by the bisulfite method. Table 2 shows the results, with an average recovery of 98.0%. Recovery is amount found divided by the amount originally present plus the amount added.

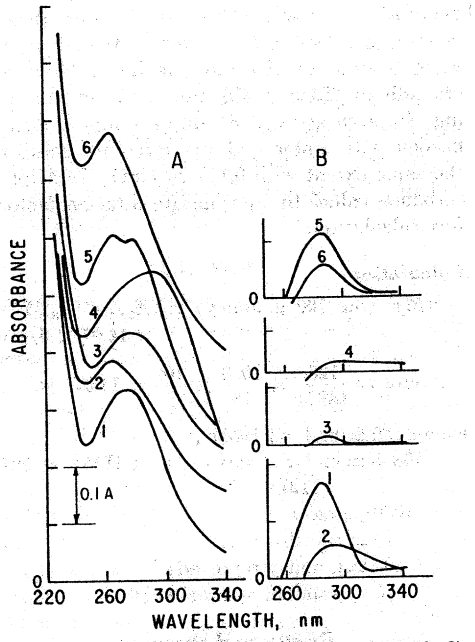

FIG. 5—A, absorption spectra of Samples 1–6 (Table 3), prepared for Winkler UV method. Baselines displaced vertically for clarity. B, difference spectra of same samples (2 g samples) prepared according to bisulfite method. Baselines as indicated.

Precision

A sample was analyzed by the bisulfite procedure 8 times in 3 days. The average was 1.30 mg HMF/100 g and the standard deviation was 0.075.

Comparison of Methods

Forty samples of honey were analyzed for HMF by the procedure described here. They

Table 2. Recovery of hydroxymethylfurfural added to various honeys (mg/100 g)

	Hydroxymethylfurfural				
No.	In honey	Added	Total present	Found	Rec., %
1	2.80	0.79	3.59	3.55	98.9
2	0.66	0.79	1.45	1.39	95.9
3	0.63	0.79	1.42	1.47	103.5
1	2.80	13.89	16.69	16.30	97.7
2	0.66	13.89	14.55	13.92	95.7
3	0.63	13.89	14.52	14.06	96.8
4	0.34	1.01	1.35	1.35	100.0
5	1.87	1.01	2.88	2.75	95.5
6	1.26	1.01	2.27	2.27	100.0
4	0.34	8.10	8.44	8.16	96.7
5	1.87	8.10	9.97	9.63	96.6
6	1.26	8.10	9.36	8.89	95.0

Table 3. Comparison of methods for hydroxymethylfurfural in honey (mg HMF/100 g honey)

No.	Winkler UV[a]	Winkler toluidine	Bisulfite
1	4.80	2.04	2.80
2	2.74	0.28	0.66
3	3.26	0.89	0.63
4	5.51	0.36	0.34
5	5.42	1.53	1.87
6	0.68	1.28	1.26
7	10.9	6.03	7.73
8	15.6	11.5	12.0
9	17.7	12.8	14.6
10	6.45	5.09	4.78
11	0.00	0.52	0.10
12	0.66	0.47	0.10
13	1.12	0.14	0.06
14	2.67	0.28	0.15
15	11.8	8.72	8.82
16	38.3	28.0	30.2
17	3.29	2.77	2.25
18	4.68	2.87	3.01
19	24.1	19.1	20.5
20	1.58	1.07	0.60
21	3.99	1.51	1.45
22	0.75	0.60	0.00
23	0.33	0.13	0.00
24	5.41	5.21	4.61
25	6.01	5.12	5.14
26	9.53	8.09	8.17
27	15.4	14.3	14.1
28	1.45	0.71	0.86
29	1.16	0.52	0.20
30	0.00	0.00	0.00
31	0.32	0.00	0.00
32	4.24	0.00	0.06
33	1.44	0.21	0.30
34	1.00	1.43	1.30
35	5.20	0.58	0.47
36	0.85	0.23	0.00
37	10.3	7.75	7.49
38	34.2	20.2	23.8
39	10.8	9.20	8.69
40	16.2	11.5	13.1
Mean	7.25	4.83	5.05

[a] Calculated with factor of 40.0 rather than Winkler factor of 43.1 (10).

had previously been analyzed by the Winkler chemical and the Winkler UV methods. They were selected to provide a wide range of HMF concentration. The results are shown in Table 3.

The 3 methods were compared by analyzing the differences between the methods for each sample. The results of 3 paired t-tests showed no evidence of a statistically significant ($P = 0.05$) difference between the toluidine method and the method described here, as shown in Table 4. Results from each of these 2 methods were significantly ($P = 0.001$) different from those by the Winkler UV method. The new method therefore appears to have met the objective for accuracy; it was submitted for collaborative study in 1978.

Factors Contributing to Empiricism

In the new method it is assumed that all of the absorbance of HMF at 284 nm in the honey solution is eliminated by bisulfite; hence the literature value for HMF absorptivity is used for calculation. In aqueous solution this is not strictly true (Fig. 1B); an average of 5.7% remains. The contribution of $NaHSO_3$ in the reference honey solution is not known exactly and is not compensated for. In aqueous solution the absorbance of 0.1% $NaHSO_3$ is negligible at 336 nm, and about 0.014 at 284 nm.

The absence of bisulfite in the sample when measured should thus lead to slightly lower values. However when a solution of glucose and fructose at final concentrations equivalent to those of honey in the procedure is clarified and analyzed, absorbance at 284 nm is 0.005, and at 336, 0.002. A degree of empiricism thus remains in the method, with essentially compensating contributions to the baseline. The difference spectra in Fig. 4, of honeys with little or no HMF, verify that no serious error is introduced.

Each of the spectra for the honey solutions in Fig. 3 was determined with the appropriate reference. Their difference at 284 nm is 0.083. The value at 284 nm in the difference spectrum is 0.087. If this value is increased by 0.014 to compensate for the lowered baseline caused by having bisulfite in the reference solution only, and then is decreased by the value at 336 for

Table 4. Analysis of data from methods comparison (Table 3)

Pair	Mean diff.	s_D	$s_{\bar{D}}$	t	Sig.[a]
Winkler toluidine vs. bisulfite	0.23	0.873	0.138	1.67	>0.05
Winkler toluidine vs. Winkler UV	2.42	2.89	0.456	5.31	<0.001
Bisulfite vs. Winkler UV	2.19	2.17	0.343	6.38	<0.001

[a] $t_{0.05} = 2.02$, 39 DF; $t_{0.001} = 3.54$, 39 DF.

nonspecific absorbance, the result is 0.085, in agreement with that obtained by subtraction. Since it is procedurally simpler to measure honey without bisulfite vs. honey with bisulfite, rather than each against a proper blank and subtract the appropriate absorbance values, the former procedure was chosen, with knowledge of very small inaccuracies.

The average for all 23 recovery determinations in Tables 1 and 2 is 97.5%. Table 3 indicates that for 40 honeys, values by the new method averaged 104.6% of those by the Winkler chemical method. Since addition of an empirical factor to compensate for the slightly low recovery would further widen the difference, it is not included. It may be noted, when considering the absolute accuracy of the chemical method, that Winkler obtained an average recovery for added HMF of 101.6% for 12 samples, with a range of 94–111% (6). The agreement between the 2 methods is considered satisfactory without further attempts to correct for unknown effects of bisulfite on honey constituents, contribution of bisulfite to baseline, or the incomplete elimination of A_{284} of HMF by bisulfite seen in pure solutions. Measurement of HMF by this or any method cannot alone differentiate between heat- or storage-abused and adulterated honey unless very high values (>50 mg/100 g) are obtained. Values above 20 mg/100 g require other confirmatory information, as will be discussed in a subsequent report.

Acknowledgments

Determination of HMF by the toluidine-barbituric method for most samples in Table 3 by James Siciliano, Eastern Regional Research Center, is acknowledged with appreciation, as is the counsel of John G. Phillips, Consulting Statistician, Northeastern Region, Federal Research, Science and Education Administration, U.S. Department of Agriculture.

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